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Chapter 12

A Different Approach for Cellular Oncogene Identification Came from *Drosophila* Genetics

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Cancer is a genetic disease

Cancer is a major health concern of our time, being responsible for more than 25% of deaths worldwide. The past two decades have produced strong evidence for the genetic basis of cancer. Cancer develops as a clonal disease occuring by the accumulation in multiple steps of genetic (or epigenetic) changes in oncogenes, tumor suppressor genes and "guardian" genes that support expansion of a new clone over the old one. Subsequently, it is the natural selection which helps expansion of a new clone carrying characteristics advantageous for proliferation [1, 2, 3].

Nevertheless, the events contributing to cancer are not restricted to the cancer cells. The most encountered example is the case of *NFkB* (nuclear factor kappa-light-chain-enhancer of activated B cells) which is a protein complex that controls the transcription of DNA, that may be up-regulated in hepatocytes through different changes in the expression of *TNF* (Tumor Necrosis Factor) from the neighboring stromal inflammatory cells, and thus can become a key contributor in many cancer cells [3].

Recently, it has been estimated that up to seven rate-limiting genetic / epigenetic events are needed for the development of a common human cancer [4]. These can appear in multiple different combinations depending on which particular tissue or cell-specific "anticancer" barriers are to be circumvented.

A lot of knowledge about cancer was obtained from studying rare familial "monogenic" cancer syndromes. Although the most of the cancer cases appear to be "sporadic", when cancer- causing gene mutations occurred only in adult somatic cells, these cases also proved to be important for understanding this intricate disease. Other important aspect refferes to the fact that many key molecular factors to cancer progression may not be deregulated at the

gene level. Downstream signaling proteins may become up-regulated by alterations in upstream growth factor signaling, altered catabolism, genes inactivated by epigenetic factors, protein expression altered by enzyme activity, degradation, chaperones, etc [4].

The idea that genes determine the growth behavior of a cell is now widely accepted, with abnormal growth reflecting the action of abnormal genes. The transformation of a normal cell to a cancer cell starts with changes in growth regulatory genes, and in the course of tumor progression further escape from normal growth control is caused by additional alterations in genes that direct cell multiplication and cell survival. Therefore, either somatic or germline mutations are both considered the root cause of cancer. These heritable changes in cancer cells are subsequently the targets for current attempts to develop effective and specific therapies for this disease [2]. As a general rule, one can say that most things related to cancer are a matter of timing and are determined by many other factors like the cell of origin, the mutations accumulated and the environment, together referred to as the molecular "road map" leading to cancer.

At this time, more than 1% of all human genes are believed to be "cancer genes". Approximately 90% of them represent somatic mutations in cancer, 20% bear germ-line mutations that predispose to cancer and 10% show both somatic and germ-line mutations [1]. Therefore, it was assumed that there are far fewer "pathways" implicated in cancer than genes. The identification of disease-related genes has led to a number of available genetic tests that detect disease or an individual's risk of disease. Gene tests are available for different disorders and also in cancer testing, some good examples being for the *BRCA1* genes related to the breast cancer, or for MEN1 and RET genes which are linked with endocrine tumors. Once more disease linked-genes are discovered, more gene tests are expected to become available [1].

1.1.1. The origin of cancer

Cancer had been recognized throughout recorded history and was known to the ancient Egyptians, from around 1600 BC, but it was not studied until the seventeenth century, when the formal study of cancer (oncology) was first documented [1]. Nevertheless, only rather recently were registered spectacular progresses in describing the fundamental molecular basis of cancer, following the entry of molecular biology and especially of genetics.

Usually, cancer is behaving exactly as a clonal disease, beginning with a mutational episode in a single cell and then develops in multiple stages through the acquisition of further mutations which are inherited through division, by the progeny of that cell. As the same outcome can also arise by epigenetic factors that alter chromatin structure, without altering the coding DNA, mutations are not the only way in which a cancer cell acquires inactivation or activation of a key gene/ protein. Therefore, sometimes the term "epimutations" is used to encompass both major routes by which cancer cells acquire aberrant expression/activity of key genes and proteins.

The adult human has been estimated to contain in average as many as 10^{14} cells, most of which could theoretically become a cancer cell given the right sort of genetic (mutations)

and epigenetic changes. Replicating cells may be most vulnerable to cancer-causing mutations. Even if some cell type, of which adult nerve cell are good examples, may avoid becoming cancer cell because they are essentially non-proliferating in the adult, most cells either regularly do or can at a pinch replicate [1]. It seems that most adult cells survive on average for 4-6 weeks and then have to be replaced. Also, over a thousand billion cells may die each day and are renewed either by replication of existing cells or from stem cells precursors. Given that every cell gets a significant amount of daily DNA damage and 10^{11} or more of them will replicate each day- that is a lot of potential cancer cells. Keeping this in mind a cancer might be expected to be a frequent incidence although so far this only happens in 1 in 3 people and usually even then only after 60 or 70 years of potentially mutation-causing events [1].

It was well documented that there is a geographical variation in cancer incidence and death, and this likely reflects socioeconomic factors. The different roles of genetic predisposition, gene-environment interaction and infectious agents shared importance in causing cancer. Recent research points to the considerable overlap between the behavior of cancer cells and that of cells during normal physiological wound healing and during embryogenesis. Similarities refers to replication, less differentiated state, invasion/migration, with the major differences reflecting the lack of control and the unscheduled nature of replication which characterizes cancer. One intrigued question addressed later was how the organism is able to distinguish between normal growth and tissue repair (normal cell cycle) on one hand and neoplastic growth (cancer cell cycles) on the other. Several theories [2, 3] sustain that for the initial expansion of a clone of cells more than one mutation is needed. Efforts in several science laboratories sustain that in certain cases the mutational route to cancer may be rather short (in molecular terms) with as few as two interlocking mutations required for initiation or progression of cancer- especially in animal models, unlike in man where at least one of these lesions involves particularly “dangerous” oncogenes such as c-*myc*. Therefore, it is believed that at least in some cases the genetic basis of a given cancer may be remarkably simple. Reference genome for man and other model organisms from the last decade has helped the explosion of new knowledge in human genetics.

1.2. The origin of oncogenes

An oncogene is a gene that contributes to converting a normal cell into a cancer cell when mutated or expressed at abnormally-high levels. Although the discovery of the origin of oncogenes came in parallel with the study of retroviruses, known in general as cancer inductors, not all retroviruses are tumor viruses [4, 5]. Even from early 1972 many researchers set out to explore the "oncogene hypothesis" proposed by Robert J. Huebner and George J. Todaro of the National Cancer Institute [6]. Looking for one mechanism to explain the induction of cancer by many different agents, Huebner and Todaro had suggested that there are the retroviral oncogenes as part of the genetic baggage of all cells, perhaps acquired through viral infection early in evolution. They supposed that the oncogenes would be innocuous as long as they remained quiescent. When stimulated into activity by a carcinogenic agent, however, they could convert cells to cancerous growth. It was reasoned

that if the hypothesis was correct, the *src (sarcoma)* gene might be found in the DNA of normal cells. The copy of *src* could therefore be identified in the DNA from uninfected chickens and other birds. The next purpose was to find DNA related to *src* in mammals, including human beings, and in fishes. All vertebrates revealed to possess a gene related to *src*, and the oncogene hypothesis was consequently declared to be correct. But, on closer inspection, however, the gene that was discovered in vertebrates proved not to be a retrovirus gene at all. It was a cellular gene, which is now called *c-src*. The most convincing evidence for this conclusion came from the finding that the protein-encoding information of *c-src* is divided into several separate domains, called exons, by intervening regions known as introns. A split configuration of this kind is typical of animal-cell genes but not of the genes of retroviruses. Apart from their introns, the versions of *c-src* found in fishes, birds and mammals are all closely related to the viral gene *v-src* and to one another. It appears the vertebrate *src* gene has survived long periods of evolution without major change, implying that it is important to the well-being of the species in which it persists [6]. As a result, the genetic view of cancer-genes has for a long time its origins in virology. Retroviral oncogenes constitute the bridge between virus-induced tumors and tumors of all other etiologies: a cellular oncogene activated by viral transduction is a mere special example of the general phenomenon of genetic alterations that can convert important and useful growth regulators of the cell into driving forces of unbridled growth. Studies on tumor viruses had shown that viral genomes could carry individual genes that, when expressed in host cells, are both necessary and sufficient for the induction of oncogenic transformation. Such oncogenes became particularly interesting in retroviruses, because they turned out to be recent acquisitions from cellular genomes, pieces of host genetic information that were mutated, transduced and expressed as part of the viral life cycle.

It was thus supposed that the biologically active cellular oncogenes are mutant forms of normal proto-oncogenes that differ in the regulation of their expression or in the structure and function of their gene products. Nevertheless, biologically active cellular oncogenes were also identified by the ability of tumor DNAs to induce transformation in gene transfer assays. Such experiments have led to the identification of more than a dozen distinct human oncogenes that are activated either by point mutations or by DNA rearrangements in human neoplasm, or by DNA rearrangements that occur in the process of gene transfer. Both somatic mutations and DNA rearrangements of such oncogenes activated in human tumors suggest implication of cellular oncogene activation in the pathogenesis of human cancers. Accordingly, in the last decade, many researchers focused their attention to studies extremely helpful in elucidating these questionable aspects by implying model organisms.

1.2.1. Classification of cancer-genes

The genetic injure present in a parental tumorigenic cell, if not correctable, is maintained such that it is a heritable trait of all subsequent generations cells. Most, if not all cancer cells contain genetic damage that appears to be the responsible event leading to tumorigenesis. Two types of genetic damage are generally found in cancer cells:

A. Dominant genetic damage- and the involved genes have termed proto-oncogenes

A proto-oncogene is a gene whose altered protein product has the capacity to induce cellular transformation. The distinction between proto-oncogene and oncogene relates to the activity of the protein product of the gene. Therefore, an oncogene is believed to be a gene that has sustained some genetic damage and produces a protein capable of cellular transformation. The process of activation of proto-oncogenes to oncogenes can include retroviral transduction or retroviral or transposon integration, point mutations, insertion mutations, gene amplification, chromosomal translocation and/or protein-protein interactions. In general, proto-oncogenes have been classified based upon sequence homology to other known proteins or based on their normal function within cells [6]. As predicted, proto-oncogenes have been identified at all levels of the various signal transduction cascades that control cell growth, proliferation and differentiation. A common rule ascertains that proto-oncogenes which were originally identified as inhabitants in transforming retroviruses are designated as *c-* indicative of the cellular origin as opposed to *v-* to signify original identification in retroviruses. The list of proto-oncogenes identified to date is rather lengthy [3, 6].

B. Recessive genetic damage- and the involved genes are variously termed tumor suppressors/ growth suppressors/ recessive oncogenes or anti-oncogenes

There is an equally important category of cancer genes that contribute to tumorigenesis through a loss of function named tumor suppressor genes. In contrast to the growth stimulatory oncogenes, tumor suppressors normally function as attenuators and as inhibitors of growth. This category includes any gene that has the potential of becoming a constitutive growth stimulator and determinant of oncogenic cellular properties. The oncogenicity of these genes is therefore correlated with a gain of function.

The normal versions of both oncogenes and tumor suppressor genes serve in diverse regulatory systems of the cell. Most proto-oncogenes encode for components of signal transduction pathways that convert an extracellular stimulus into a programmed pattern of gene expression. A functional relatedness of tumor suppressors is less apparent; some domains of tumor suppressor gene action include cell surface properties, signal transduction, gene transcription, DNA repair, and checkpoints for cell division [6].

1.3. Identification of new target cancer-related genes by insertional mutagenesis

Insertional mutagenesis is a good mechanism for identifying new cellular proto-oncogenes, especially when correlating with neoplasms induction by different viruses or transposons. The activation of a candidate oncogene by such an insertion not only provides a means of identifying such potential oncogenes, but also permits their isolation as molecular clones for subsequent investigation. After integrating its viral/ transposon DNA, the virus/ transposon itself represents a marker that could help to isolate surrounding molecular clones. These

clones can furthermore provide the flanking genomic sequences, which can reveal the targeted proto-oncogene activated/ targeted by the insertion. A good example for this mechanism is provided by *wnt-1* (previously called *int-1*) which is usually activated upon integration of MMTV proviral DNA in mouse mammary carcinomas, first isolated in the laboratory by Harold Varmus in 1982 [7]. The MMTV virus LTR end usually acts as an enhancer to elevate *wnt-1* expression. The flanking DNA could have be cloned and then sequenced and it helped to elucidate the targeted *wnt-1* gene sequence. So it was revealed that the *wnt-1* genomic locus contains a transcriptional unit that was activated in tumors, either by upstream or downstream MMTV DNA insertion. The *wnt*-1 thus appeared to be a cellular oncogene because of its frequent activation by insertional viral spontaneous mutagenesis. But the designation of *wnt-1* as a real oncogene was later decided when it was shown that introduction of this gene into cultured mammary epithelial cells induced abnormal growth characteristic of neoplastic transformation [8]. Although it was initially identified by indirect criteria of structural alteration in tumors, this demonstration of the biological activity of *wnt*-1 provided important subsequent justification for this approach for oncogenes isolation. Provirus insertional mutagenesis was also implicated in activation of other cellular proto-oncogenes, encoding for different growth factors. For example, the gibbon ape leukemia cell line *MLA144* constitutively produces the *interleukin*-2 growth factor (T-cell growth factor), which appeared to be required for proliferation of these cells. Subsequent analysis of the interleukin-2 gene revealed that its constitutive expression is a consequence of the integration of gibbon ape leukemia virus DNA in the 3′ untranslated gene region [8].

Most recently studies revealed that one of the best examples of how a transposon insertion could facilitate for oncogene/ candidate-cancer gene identification is given by numerous studies from *Drosophila melanogaster*. Using genetic methods in *Drosophila* null alleles of the *P*-transposon tagged genes can be generated by remobilizing the transposons and screening for transposon's imprecise excisions. Different studies have also highlighted the power of *D. melanogaster P*-derivative transposons for examining cooperative interactions between tumor suppressors and oncogenes and for generating *in vivo* models of tumor development and metastasis [9]. Therefore, *Drosophila* is currently widely used as a model organism to explore the functions of different genes particularly those ones which are structural homologs of human oncogenes and tumor suppressor genes involved in a variety of tumors.

1.4. How similar are the fruit flies and humans

The fundamental aspects of the cellular biology, regulation the gene expression, neuronal connectivity, synaptogenesis, cellular signaling and cellular death are commonly accepted as being similar between humans and fruit flies. The structural homology between *Drosophila* and human genes has been revealed immediately after sequencing the *D.melanogaster* genome [10], and the human genome. Thereafter, the interest was focused on considerable studies using *Drosophila* as an experimental model for different human diseases. Numerous studies have been done to identify genes implicated in human pathologies that could be investigated in Drosophila. The most detailed study was done by

Reiter and collaborators [11] in 2001. Using the Online Mendelian Inheritance in Man (Homophila) database, Reiter and coworkers found that 714 of the 929 (77%) OMIM human disease gene entries have highly similar cognates in *Drosophila*, which were referring hereafter as "related genes". These data can be accessed through http://superfly.ucsd.edu/homophila, and the query can be made by key word, disease name, fly gene, and OMIM number (Table 1).

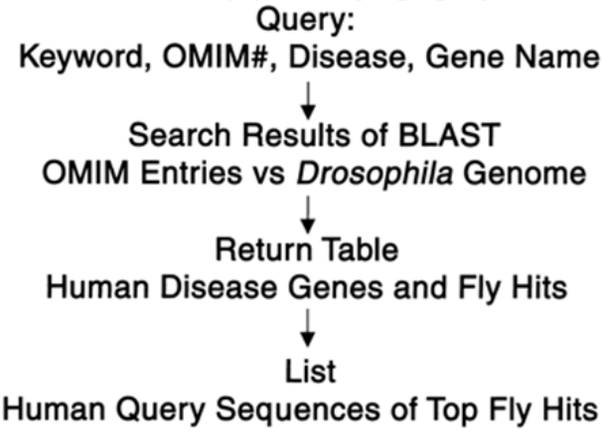

Table 1. How to query the Homophila database. The user enters the text query in the form of human disease name, OMIM number, fly gene name, or keyword search through the human disease entry box. A window with information on the disease name, and human and fly genes that match the key word query is opened. The user then can examine the details of an individual human-to-*Drosophila* BLAST comparison to get more information on the specific BLAST score, alignment, and other hits to this gene. In addition, transposons' (*P*-element) information is found at this level [after 11].

A list of disease phenotypes resulting from mutations in genes that are highly related to *Drosophila* genes has been categorized into various subclasses based on clinical phenotypes. A large number of human disease genes sharing *Drosophila* counterparts involved in different disorders such as: cancer, non-myelin associated neurological disorders, other developmental defects etc, has been therefore identified. Additional notable result of their study was that the great majority of *Drosophila* genes related to human disease genes (e.g. 395 genes out of 548) had not been analyzed by loss-of-function genetics by that time. Moreover, they found that many of these *Drosophila* related counterpart genes are marked by *P*-elements insertions in or near them (e.g., within 1 kb of the gene-coding region) and the *P*-element insertions were the only known alleles of those genes. Immediately after this study was published, using routine genetic methods in *Drosophila*, the possibility to create null alleles of these 56 *P*-element tagged genes was starting in different laboratories by remobilizing the *P*-elements and screening for imprecise excisions that delete all or parts of the coding regions. Thus, loss-of-function analysis has become possible for identifying the function of these "related genes". Without the complete comparisons of the genomes in a database like *Homophila*, it would not be immediately obvious that genes e.g. responsible for human deafness could be functionally analyzed in an organism like *Drosophila* [11]. Surprisingly, the human genome is predicted to have only little over twice the number of genes found in flies and a comparison of both proteomes indicates 67% similarity at the amino acid level [10].

2. Why studying cancer in the fruit flies?

The expansion of human cancer is a multistep route, involving the cooperation of mutations in signaling, cell-cycle and cell-death pathways, as well as interactions between the tumor and the microenvironment. In this context, to *in vivo* explore the steps of tumorigenesis, simple animal models are needed. The genetically amenable, multicellular organism, the vinegar fly, *D.melanogaster*, can be used to elucidate the functions of different human structural homologues whose mutations were identified in different types of cancers [12]. This choice is not surprisingly taking into account the research history in *Drosophila* and the contributions of the *Drosophila* genetics for understanding the signaling pathways implicated in oncogenesis, such as: *Ras/MAPK, Notch, Wnt/wingless, hedgehog* and *BMP*.

D. melanogaster is an experimental model organism currently largely used for cancer research [13]. In 1916, decades before *Drosophila* would become one of the most popular models for studying many aspects of modern biology, the discovery of melanotic tumor-like granules in mutant larvae, by Bridges and Stark, first suggested that flies could develop tumors [14]. It took more than 50 years of genetic analysis to obtain convincing data which prove that insects can suffer from cancer [15]. Later, spontaneous mutations were identified causing the death of the animals at larval stages, because of overproliferation of certain internal tissues [16, 17]. Another study [18] showed that homozygous mutations in a series of genes from *Drosophila* can cause the appearance of tissue-specific tumors, which can affect either the embryonic or the larval development. Among these genes, the *lethal (2) giant larvae* (*l(2)gl*), has been the most studied. Homozygous mutations in *l(2)gl* produce malignant tumors in the brain and the imaginal discs. The *l(2)gl* gene was cloned, introduced back into the genome of l(2)gl-deficient animals and shown to restore the normal development [19], a process called "rescue-phenotype". A mosaic screen for over-proliferation mutants has been used successfully to identify several novel tumor suppressors in flies, including the large tumor suppressor (*lats*; also known as *wts*) gene. Somatic cells mutant for *lats* undergo extensive proliferation and form large tumor outgrowths with morphological characteristics similar to those of human tumors, confirming that *Drosophila* can grow tumors that are comparable with those found in humans. The human homolog of the *lats* gene (*LATS1*) could be used to suppress tumor growth and rescue developmental defects in *lats* mutant flies, including embryonic lethality [20]. Studies in *Drosophila* of such genes provided information that was directly relevant to tumorigenesis in humans.

In 2009, the second BioMed Conference from Barcelona entitled "Modelling Cancer in *Drosophila*" emphasizes again *Drosophila* as a model to elucidate human cancers. It was for the first time when a group of scientists came together to discuss the ways in which the fruit fly could provide novel contributions to the field of human cancers. For some of the presentations, discoveries in *Drosophila* were later validated in mammalian system or in humans [21]. So that, there is a large spectrum of candidate genes implicated in human pathologies that can be studied in *Drosophila*, and the lack of redundancy can simplify the analysis of biological process in the fly [22]. The functional orthology between *Drosophila* and human genes can be proved by rescue phenotype experiments (the equivalent of gene therapy) and it is working and gives surprising results.

2.1. Testing for preserved function between Drosophila genes and human counterparts

There are a number of ways to test the function of a foreign gene/ protein in transgenic *Drosophila*. Homologs of a fly gene for which mutants exist can be tested for the ability to rescue the fly mutant phenotype. If the fly counterpart has dominant effects or if one might expect dominant effects as a result of the function of the protein in vertebrates (such as for a dominant oncogene or disease gene), then another test is to determine whether the vertebrate homolog can induce similar dominant phenotypes in flies. There are examples of dominant oncogenic mutations leading to a form of the protein that also functions dominantly in the fly [23]. In some cases, expression of vertebrate/human genes in fruit flies has demonstrated that a conserved function of the vertebrate and fly genes is autoregulation; thus, the vertebrate protein (frequently a transcription factor) turns on expression of the endogenous fly counterpart. If one has mutants in the fly gene involved. Then, it is possible to test for functional conservation in the genetic background of a protein null mutant of the fly gene and, hence, address broader aspects of functional conservation.

Herein are presented some results of a study concerned on the clarification of the putative functional conservation between *DmManf* gene from *Drosophila* (previously called *ARP-like* for *arginine-rich protein-like*), and its counterpart- the human *Manf* gene (*Mesencephalic astrocyte-derived neuroptrophic factor*), which was found to be implicated in various human pathologies, including cancer.

2.2. Exploration of the DmManf in comparison with Manf putative oncogene from human

Mesencephalic astrocyte-derived neurotrophic factor, *DmManf* gene is referred in *FlyBase* by the symbol *CG7013* (*CG*, computed gene). It has the cytological map location 89B19, in the right arm of the 3rd chromosome. The molecular function is unknown. Previously, it was shown that a *P{EP}* transposon insertion at the position –157 upstream of the 5'UTR region of the *Manf* gene does not affect the *Manf* gene function [24].

The *DmManf* gene contains 1436 nucleotides and encodes for a protein of 173 amino acids. In the fruit-fly stock *EP(3)3171* the *DmManf* gene is associated with a *P* derivative transposon. In general, transposable elements insertion's are extremely powerful means of gene disruption. The transposon associated with *DmManf* gene was symbolized by *P{EP}EP3171* and was first inserted in the 5'UTR region of *DmManf* gene, and then, could be mobilized generating mutant alleles of the targeted gene.

The human *Manf* counterpart gene, previously named *ARP* (from Arginine-Rich Protein) is located in the chromosomal band 3p21.2, a region that is frequently deleted in a variety of solid tumors. It encodes for a protein highly conserved in evolution. First oncological information was given by Shridhar and coworkers [25, 12] who reported an ATG-to-AGG transversion in codon 50 of the *ARP* gene or deletion of codon 50 in different tumor types including 10 of 21 sporadic renal cell carcinomas. Later (1997), they observed the same

mutations in 11 of 37 pancreatic tumors. Either of the changes abolishes a methionine residue and gives rise to an uninterrupted string of AGG trinucleotides in the *ARP* gene and arginines in its predicted protein product. The finding of 4 other nucleotide substitutions in codon 50 that replaced methionine with 4 different amino acids other than arginine suggested that loss of this methionine residue is critical to a carcinogenic role of this gene.

Their finding of an AGG-to-AAG (arg-to-lys) mutation in the adjacent codon 51 in 2 tumors emphasized further the importance of this region. Other evidence [25] suggested that only a single copy of the *ARP/Manf* gene is mutated in the cancer cells, indicating its possible causal role as an oncogene.

Mesencephalic astrocyte-derived neurotrophic factor sequences are referring to a family of small proteins of approximately 170 residues which contain four di-sulfide bridges that are highly conserved, from nematodes to humans (Table 2).

	Genes	**Proteins**
1	MANF, *H.sapiens* mesencephalic astrocyte-derived neurotrophic factor	NP_006001.3 182 aa
2	MANF, *P.troglodytes* mesencephalic astrocyte-derived neurotrophic factor	XP_001169644.1 246 aa
3	MANF, *C.lupus* mesencephalic astrocyte-derived neurotrophic factor	XP_850540.1 179 aa
4	MANF, *B.taurus* mesencephalic astrocyte-derived neurotrophic factor	NP_001094681.1 179 aa
5	Manf, *M.musculus* mesencephalic astrocyte-derived neurotrophic factor	NP_083379.2 179 aa
6	Manf, *R.norvegicus* mesencephalic astrocyte-derived neurotrophic factor	XP_236614.3 179 aa
7	Manf, *D.rerio* mesencephalic astrocyte-derived neurotrophic factor	NP_001070097.1 180 aa
9	Manf, *D.melanogaster* Mesencephalic astrocyte-derived neurotrophic factor	NP_477445.1 173 aa
10	AgaP_AGAP003016, *A.gambiae* AGAP003016-PA	XP_311862.3 180 aa
11	Y54G2A.23, *C.elegans* hypothetical protein	NP_500273.2 168 aa

Table 2. HomoloGene report showing the putative homologs of mesencephalic astrocyte-derived neurotrophic factor. The identification number of the protein, the size and their conserved domain architectures are are assigned (www.ncbi.nlm.nih.gov/homologene).

The amino acid sequences of this highly conserved protein in evolution shows 51% identity on average between *Drosophila Manf* gene and the human *Manf* (Fig. 1).

The higher homology revealed by the comparison of the whole aminoacid sequences of the *Drosophila* and human proteins (51% identity, 71% positives) as compared to the amino acids of the Saposin_like domains (41% identity, 58% positives) suggests that, in addition to the Saposin_like domain, there are other motifs which highlight the similarity or even identity

between the compared aminoacid sequences. These motifs could be important for a particular function of the protein and suggest a similar function of the two proteins. This remark is in agreement with a similar situation that was encountered when *Drosophila lats* and human *LATS1* were compared [20]. Although the overall sequence similarity in the amino-terminal regions of these two proteins was lower (22% identity and 42% similarity) than in the kinase carboxy-terminal (74% sequence identity), stretches of highly conserved sequences have been identified in both proteins, and finally, they proved that the genes are even functionally conserved. Another similar study presents the case of *Mgstl* which encodes a protein similar to human *mGST*. Because the identity between both genes was 45% but their hydrophobic profiles were also very similar, the authors' expectation was that these genes share a functional similarity [26].

```
 Score =  174 bits (442),  Expect = 1e-59, Method: Compositional matrix adjust.
 Identities = 83/163 (51%), Positives = 116/163 (71%), Gaps = 1/163 (1%)

Query  10   LAVALALSVLPGSRALRPGDCEVCISYLGRFYQDLKDRDVTFSPATIENELIKFCREARG  69
            + +    ++   S AL+  DCEVC+  + RF   L D         IE    KFC+  +
Sbjct  8    VVIGFLATLAQTSLALKEEDCEVCVKTVRRFADSLDD-STKKDYKQIETAFKKFCKAQKN  66

Query  70   KENRLCYYIGATDDAATKIINEVSKPLAHHIPVEKICEKLKKKDSQICELKYDKQIDLST  129
            KE+R CYY+G  +++AT I+NE+SKPL+  +P EKICEKLKKKD+QIC+L+Y+KQIDL++
Sbjct  67   KEHRFCYYLGGLEESATGILNELSKPLSWSMPAEKICEKLKKKDAQICDLRYEKQIDLNS  126

Query  130  VDLKKLRVKELKKILDDWGETCKGCAEKSDYIRKINELMPKYA  172
            VDLKKL+V++LKKIL+DW E+C GC EK D+I++I EL PKY+
Sbjct  127  VDLKKLKVRDLKKILNDWDESCDGCLEKGDFIKRIEELKPKYS  169
```

Figure 1. Comparison between *D.melanogaster Manf* and human *Manf* sequences. „Query"= protein from human and „Sbjct= protein from *Drosophila*. Amino acids from Saposin-like domain are surrounded by green; bordered in blue is EF-hand domain. For detailes, see text.

To study whether *DmManf* holds not only a structural, but also a true *in vivo* functional similarity with its human counterpart, we mobilized the *P{EP}EP3171*-element from the *EP(3)3171* line to obtain specific mutant alleles of the *DmManf* gene. Different mechanisms of repairing the double-strand break generated by the *P{EP}* excision induced a variety of new genetic variants, including loss-of-function *DmManf* alleles [27].

In this study we took advantage of our first reported loss-of-function mutant *DmManf* allele, namelythe $Manf^{\Delta 1151}$ (GenBank ID: DQ649527). The $Manf^{\Delta 1151}$ allele was isolated in *Ex.35* mutant line (Fig. 2). By DNA sequencing of the specific mutant amplicon we discovered that the *Ex.35* mutant line contains a deletion of 1278 bp that removes part of the intergenic region, the 5'UTR and almost the whole coding region of the *DmManf* gene, leaving behind the distal end of the last exon and the 3'UTR region (Fig.2 Ba) and is affecting only the *DmManf* gene.

The $Manf^{\Delta 1151}$ allele from *Ex.35* line proved to be homozygous lethal. To define the lethal phase during development, we took advantage of the *GFP* (*Green Fluorescent Protein*) marker present in the *Drosophila* balancer chromosome *TM3SerGFP*, which could help distinguishing,

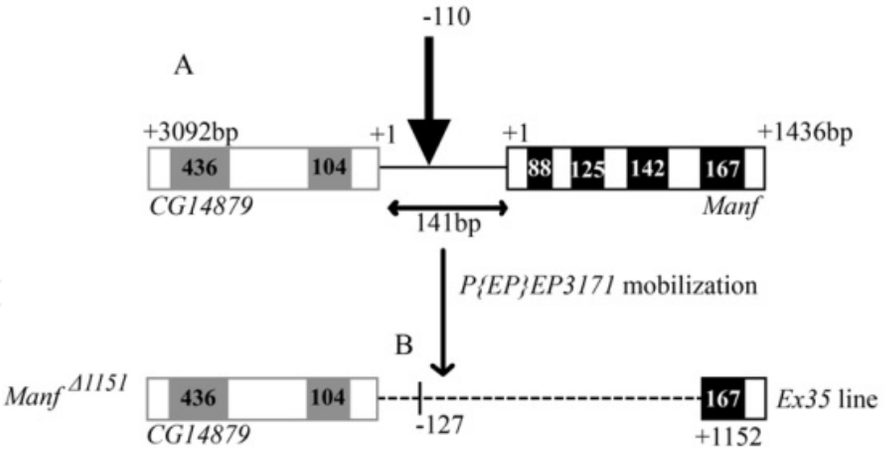

Figure 2. The structure and deletion breakpoints of *P{EP}EP3171* homozygous lethal allele found in *Ex.35* line. A: The genomic context including *Drosophila Manf* and *CG14879* genes separated by 141bp intergenic region and transcribed in opposite direction. Δ stands for *P{EP}EP3171* original insertion site. Exons are indicated by filled boxes, introns by empty boxes. Nucleotide numbers are assigned for the exons. B): The lethal $Manf^{\Delta 1151}$ alele genomic deletion is indicated by the dashed line. The numbers of nucleotides are assigned for each exon [after 27].

under an UV source, the *GFP* heterozygous individuals and *non-GFP* homozygous individuals. Therefore, after letting *Drosophila* females from *Ex.35* line to lay eggs onto Petri dishes containing appropriate culture medium, we followed the development of the embryos and larvae comparing the numbers of the heterozygous and homozygous individuals. Starting with 594 embryos, we noticed that the number of the homozygotes was continuously decreasing during the subsequent developmental stages. As it revealed, the homozygous lethality was polyphasic, the homozygotes mostly died in the embryonic and 1st instar larval (L1) phases, although L2 stage escapers were also found. Among the counted 3rd stage larvae, two *non-GFP* excapers were found. One of them was transferred onto another culture plate and observed for several days. This mutant L3 larva contained some internal „holes", *i.e.* empty, transparent spaces similar to that ''empty'' spaces found in another *DmManf* specific mutant, from a similar study [23]. First these homozygous larvae atypically wander away from the food, and then move more slowly, and finally freeze immobilized but still responding to touch. It was unable to enter the pupal stage and died keeping the phenotype described before. Homozygous mutant adults for $Manf^{\Delta 1151}$ allele were never found. These empty „holes" remained unchanged during the observation period, suggesting that the pupae died inside, probably after several unsuccessful encapsulation immune reactions by which lammellocytes should encapsulate melanise and kill the parasites, fungi or even abnormal cells- such as tumor cells (Fig. 3).

Most of the *Drosophila* overgrowing mutations are late larval or pupal lethal and affect the adult organ rudiments, imaginal discs, while leaving the other larval tissues necessary for larval survival mostly unaffected [28, 16]. The majority of such mutations display a prolonged larval period, also seen in the case of $Manf^{\Delta 1151}$mutant, which can be explained by the presence of non-differentiating growing imaginal rudiments preventing ecdysone release [29].

While *DmManf* recently [23] was found to be required during the maturation of the embryonic nervous system for maintenance of neuronal connectivity, there were also indications (http://superfly.ucsd.edu/homophila/) of a possible role of *Manf* /*ARP* in human tumor formation [12, 25].

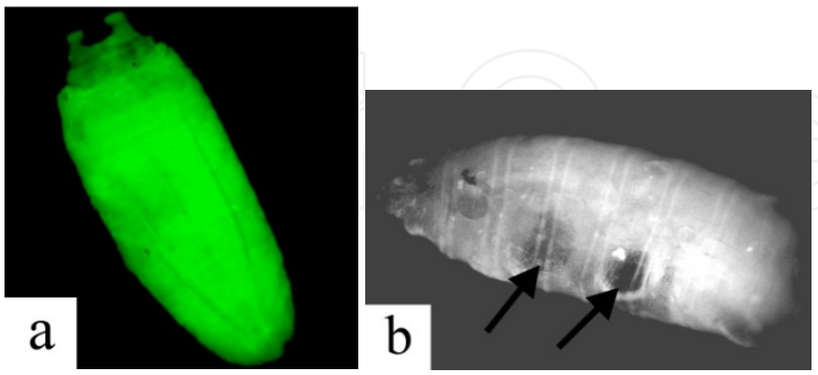

Figure 3. Heterozygote and homozygote pupae from *Ex.35* line. a) *Ex.35* heterozygous control pupae (genotype: $Manf^{\Delta 1151}$/TM3SerGFP); b) *Ex.35* homozygous pupae (genotype: $Manf^{\Delta 1151}$/ $Manf^{\Delta 1151}$); arrows show the internal holes in the dead pupa.

A wealth of data support the view that cancer is a multistage disease progressing via the accumulation of multiple genetic changes lesions that compromise the normal control of cell proliferation, survival, differentiation, migration and social interactions with neighboring cells [1]. It is also important to note that apparently phenotypically similar cancers may arise through different combinations of lesions: there are likely many different routes to cancer even in the same cell type. Many key cancer –relevant signaling pathways may be activated or inactivated by mutations at various points that could result in largely identical cell behavior.

From this point of view, interesting cancer-like phenotypes were observed for different mutants previously obtained after *P{EP}EP3171* transposon mobilization, from the *DmManf* gene vicinity [27]. The most aggressive cancer-like phenotypes which appeared in our mutants either killed the adult flies, e.g. in the case of *Ex.29* mutant (Fig. 4) or let the adults survive, although they expressed in all the body melanotic tissue/nodules, in both females and male, *e.g*. in the case of *A26.1* mutant (Fig. 6). Symbols and numbers in the mutant names designate different lines obtained from different *P{EP}EP3171* mobilization experiments [27].

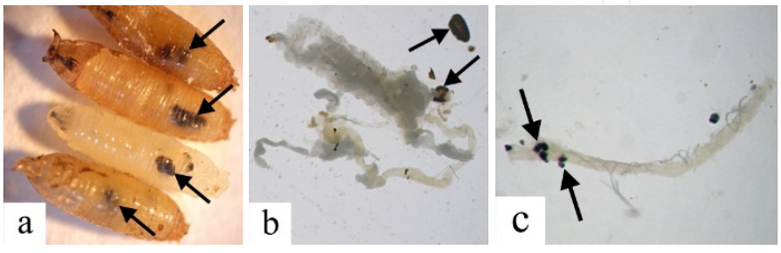

Figure 4. Larval and pupal phenotypes found in the *Ex.29* mutant. a. Larvae and pupae containing melanotic masses; b. dissected homozygous mutant larva in the III^{rd} instar enclosing melanotic masses; c. The gut surrounded by melanotic masses. The arrows label melanotic masses. The *Ex.29*/*Ex.29* homozygote mutants never developed as adults.

The appearances of the melanotic masses in flies were extensively characterized. Although the *Toll* pathway seems to be responsible for the formation of melanotic masses in *Drosophila* [30], other genes could also be implicated upstream of downstream of this well characterized pathway. Spectacular results were obtained by investigating the ultra-structure of the interesting melanotic masses found in case of *Ex.29* mutant under the electron microscope. After dissecting normal and melanotic gut of the *Ex29*/*Ex29* homozygote mutant 3rd instar larvae, several sections were examined under the electron microscope (Fig. 5). We noticed that in the cells of control normal gut the nucleus revealed a typical structure with nuclear envelope, caryoplasm and nucleolus (Fig 5a). Caryoplasma, the fundamental substance of this nucleus appeared typical, as a protein gel with the embedded chromosomes and a nucleolus. Chromatin displayed a fibrillar structure, consisting mainly of DNA associated with histones and non-histone proteins. Chromatic substance of the nucleus become visible as a network, resulting from a strong despiralization, hydration, swelling, dragging and overlapping events of the chromosomes. In some parts, the chromatic substance disclosed more condensed probably corresponding to heterochromatic areas.

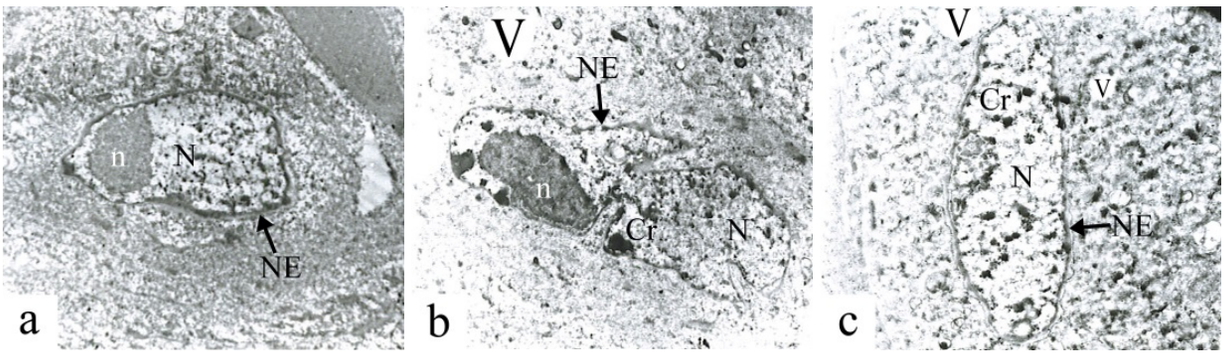

Figure 5. Electron microscopic images after micro-dissection of *Ex.29* homozygote 3rd instar larval gut; a. Nucleus and nucleolus of a normal gut cell. Magnification is 8640X; b. Dissection through melanotic "young tumor"- light black masses; the nucleus has a lobated edge, magnification 8640X; c. Dissection through melanotic "old tumor" -dark black masses, showing a nucleus without nucleolus. Magnifications 12400X. N- Nucleus; n- nucleolus; NE- nuclear envelope, V- vacuole; Cr- chromatin.

Nucleolus, another important cellular structure displayed an oval shape with irregular contour (Figs. 5a, 5b), without specific membrane, being surrounded by a network of chromatic filaments of nuclear origin which twisted into perinucleolar chromatin. As typical, is prearranged in a fibrillar-granular structure consisting of fibrils and ribo-nucleo-proteinic granules, like ribosomes.

In contrast, in the „old tumor" dark black masses, resembling senescent cells (Fig. 5c) appeared having ceased its function, many structural changes occurring probably due to the intensification of autolitic processes. As a result, the cytoplasmic content decreased significantly, most of the cellular volume being replaced by the vacuolar system. In this cell the nucleus still persists, although the nucleolus have disappeared. Only a few blocks of chromatin and an increased number of autolytic vacuoles can be detected. These changes lead to the idea that such a cell is doomed to apoptosis. Other notable melanotic-like nodule

phenotype was observed in case of the *A26.3* mutant stock, which contains *DmManf*$^{A26.3}$ allele, carrying a residual part from the original *P* element insertion (GenBank ID: HQ623183). The stereomicroscopic examination of these mutants showed mild melanotic masses in the larvae (Fig. 6).

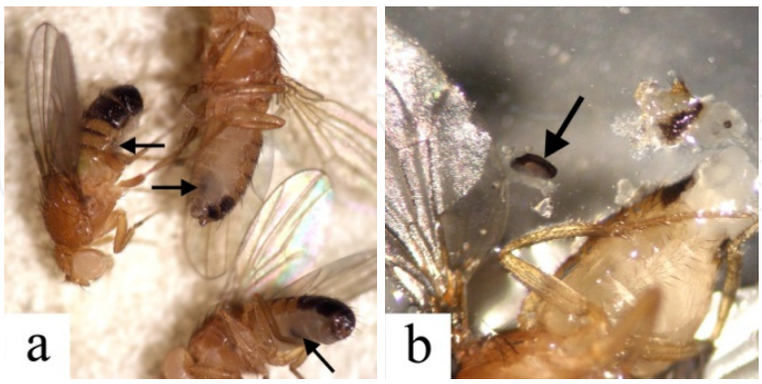

Figure 6. The *A26.3* mutant phenotype is shown. a. Melanotic nodules in the adult flies, both in males and females. b dissected adult female carrying black melanotic nodules/ pseudo-tumors.

Particular mutants expressing characteristic melanotic phenotype were also obtained in other insertional/ excisional *P* element mutagenesis experiments [31]. As it was shown previously, mutations in ~30 genes that regulate different pathways and developmental processes in *Drosophila* can cause a melanotic phenotype in larvae. The observed melanotic masses were generally linked to the hemocyte-mediated immune response. In general, the melanotic masses can be subdivided into melanotic nodules engaging the hemocyte-mediated encapsulation and into melanizations that are not encapsulated by hemocytes [31]. With rare exception, the encapsulation is carried out by lamellocytes. Encapsulated nodules are found in the hemocoel or in association with the lymph gland, while melanizations are located in the gut, salivary gland, and tracheae. These results can show that the phenotype of each mutant not only reflects its connection to a particular genetic pathway but also point to the tissue-specific role of the individual gene.

Half a century ago, melanotic tumors in *Drosophila* larvae and adults were viewed as the equivalent of cancer and as events of controlled histological differentiation that could be manipulated genetically. The participation of blood cells in the formation of some melanotic tumors was reported at about the same time [32, 33]. Black melanotic spots are found in a number of different mutants and have been called, interchangeably, melanotic tumors or pseudotumors. These ‘‘tumors’’ are usually not invasive and involve tumorous overgrowth only in some instances. Therefore it is generally accepted to use the term ‘‘melanotic masses’’ to describe the phenotype generally and ‘‘melanotic nodules’’ and ‘‘melanizations’’ to describe more specific phenotypes.

Experiments in our laboratory indicated that an artificial transposon, *P{EP}EP3171*, when mobilized [27] could induce variable mutant genotypes and phenotypes resulting in a polyallelic series of the *DmManf* and/ or other interactor genes, including melanotic masses and nodules. This is not particularly suprising, taking into account other genes, *e.g*. the deep

orange (*dor*) gene, whose poly-alellic series affect different functions resulting in lethality, male sterility, sterility with maternal effect, or simple changes in the eye color [34]. We also found *Drosophila* mutants with paternal effect sterility, which could give away *DmManf* gene's pleiotropic functions. By investigating all these isolated alleles we can reach comprehensive understanding of the role of the *DmManf* gene.

2.3. Application of the rescue phenotype (gene therapy) technique in flies

Gene therapy consists of the insertion of genes into an individual's cells and tissues to treat a disease, and hereditary diseases, in which a defective mutant allele is replaced by a functional one. Although the technology is still in its infancy, the researchers have already successfully tested it in *Drosophila*, for homozygous lethal mutations of the *l(2)gl* [19] or the *lats* gene [35], which could be saved by the orthologous genes from human: *Hugl*-1, *scrib*, *dlg* [36] etc. Therefore, when expressing a foreign gene in the fly in a tissue that normally does not express any such gene, screening of interacting proteins will be useful for understanding the function of the gene in its normal cellular context. It is important to assess whether any phenotypic effects observed in the fly accurately reflect conserved functions of the vertebrate protein under scrutiny. For example, will vertebrate anti-apoptotic genes block *Drosophila* programmed cell death? Will the vertebrate homolog, like its fly counterpart, direct ectopic tissue formation in the fly? If the vertebrate cDNA induces a dominant effect, is that effect the result of elevated levels of a normal activity of the protein (a hypermorphic effect) or of a new activity of the protein that may have little to do with its normal function (a neomorphic effect). Neomorphic effects, for example, might be the result of subcellular mislocalization of the vertebrate protein in the fly. To what degree does the pathology of a human disease gene reflect biological effects known to occur in humans or vertebrate models, and can these effects be faithfully replicated in the fly model? These are, of course, specific issues that vary for any one gene of interest, and they are critical to consider.

2.3.1. DmManf is the true ortholog of the human Manf putative oncogene

To investigate whether human *Manf* is able to compensate for the loss of function of *DmManf* mutants, we carried out rescue experiments with *UAS-HsManf* transgenic flies. We applied the gene therapy (rescue phenotype) procedure to the *Ex.35* mutant which contains the loss-of-function allele $Manf^{\Delta 1151}$, and shows lethal phenotype. First, we verified if the $Manf^{\Delta 1151}$ mutant lethality was solely the result of the *DmManf* deletion, by inducing the ubiquitous ectopic expression of *UAS-DmManf* in the $DmManf^{\Delta 1151}$ homozygote mutant background using the Actin-GAL4 driver (Fig. 7). Then, the ubiquitous Actin-GAL4 *UAS-HsManf* expression was tested if it is also able to rescue the adult lethality of $DmManf^{\Delta 1151}$ homozygote mutants (Fig.7).

We proved that the lethality was only caused by deletion of *DmManf* demonstrating that *DmManf* is important at least for the normal development and essential for viability. The rescue percentage of adult flies was similarly obtained when using separately the *Drosophila* and the Human *Manf* transgenes, driven by the ActinGal4 driver. These results demonstrate

that *DmManf* is the fruit fly functional ortholog of human *Manf*, and that fruit fly and human Manf share a yet unknown cognate receptor, essential for viability and normal development.

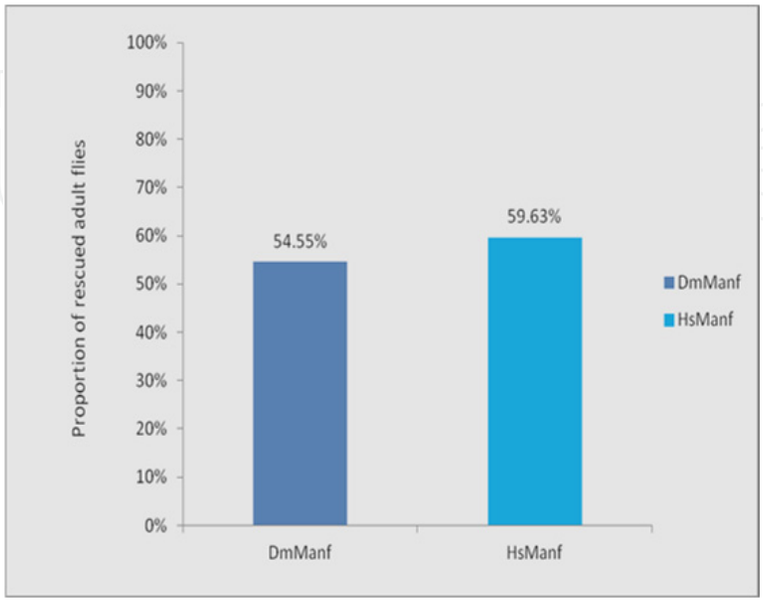

Figure 7. The lethality of *DmManf*$^{\Delta 115}$ adults is rescued by ectopic UAS-*DmManf* and UAS-*HsManf* expression with the ubiquitous Actin-GAL4 driver. The proportion of rescued adults relative to all adults is presented; 100% indicates the maximum expected value of complete rescue estimated by Mendelian inheritance.

Herein is described a pilot experiment that supports the evolutionary conservation of the *Manf* gene functions' in the fruit fly- *Drosophila melanogaster* and its important role in deciphering human pathologies. Our results complement previous results [23] when *DmManf* mutant (namely *DmManf*$^{\Delta 96}$) lethality rescue- phenotype experiment resulted in complete larval rescue up to the pupal stage [23]. These authors were using 69B-GAL4 driving the transgenic *DmManf* expression in epidermis and CNS, proving the importance of *DmManf* expression in both tissues in the fly. They also proved that *DmManf* is required for the maintenance of the DA neurites but not the neurites of serotonergic or the subpopulation of motoneurons. Surprisingly, despite the axonal degeneration in *DmManf* $^{\Delta 96}$ mutant larvae, the cell body of DA neurons persists. Moreover, some DA neurons but not their neurites persist even when their death was ectopically triggered by over expression of the proapoptotic proteins. Thus, programmed cell death in the *Drosophila* DA neurons seems to follow a ''dying-back'' pattern where the neurites degenerate first followed by the death of the cell body [23, 37]. Whether *DmManf* is a *bona fide* NTF promoting the survival of DA neurons remains, however, open as the mutant larvae died before it could be judged [23]. By TEM analysis, the elimination of *DmManf* causes cell death resembling caspase independent cell death, characterized by swelling of organelles, and the appearance of ''empty'' spaces [23, 38]. Similar observations were also found in the case of our mutant *Ex.35*, which contains the *DmManf*$^{\Delta 115}$ allele (see Fig. 3).

By the experiments involving *DmManf* gene from *Drosophila* presented here, some scientific data about the yet unknown role of this gene was obtained. We completed a first pilot phenotypic rescue of our *DmManf* deficient animals. It still remained unclear if the *DmManf* is a true oncogene or a tumor-suppressor gene. We assumed that a real cancerous phenotype could have not appeared in the *Ex.35* mutant, either because *DmManf* gene might not be a canonical oncogene, or because while *DmManf* although preserved a particular function in the cancerous process, other additional mutations were simultaneously needed for the cancerous phenotype to appear. It is accepted that for the initial expansion of a cell clone more than one mutation is usually needed. Work in several laboratories sustain that in certain cases the mutational route to cancer may be either short (in genetic terms) with as few as two interlocking mutations required for initiation or progression of cancer, or long, and these instances can be easily clarified routinely in studies which involve animal models.

3. Materials and methods

Drosophila strains: For genetic nomenclature, cytology and description of mutations and chromosomes see [39] and *Flybase* [40]. *Drosophila* strains used are: *w;EP(3)3171/TM3SbSer [24], w;TM3SerGFP/TM6TbSb, Ex.35/TM3, Ex.29/TM3, A26.3/TM3, Actin-GAL4/SM6Cy*. For the phenotypic rescue experiments, the transgenic stocks UAS-*DmManf* and UAS-*HumanManf* were received as gifts from Dr. T.I.Heino. Fly crosses were done on standard cornmeal-yeast-agar medium, at 25°C.

Phenotypic rescue experiments. The transgenic lines for UAS-*DmManf* and UAS-*HsManf* were recombined together with Actin-GAL4 driver, a GAL4 line of ubiquitous expression, on the $DmManf^{\Delta 115}$ mutant background. For each experiment 3 independent crosses were made and transferred twice to fresh vials; progeny from all vials of each cross was counted, and the proportion of rescued adults relative to all adults was calculated.

StereoMicroscopy and Image Analyses: Larval and pupal images were taken through an *Olympus SZX7* stereo-microscope equipped with an *Olympus* DP70 camera.

Electron Microscopy (EM) Analyses. The gut and melanotic tissues isolated from dissected *Ex.29* homozygous larvae were fixed in 4% glutar-aldehyde in 0.1M sodium cacodylate buffer, pH7.3, 4h at 40°C and post-fixed in 1.5 % osmium tetroxide in the same buffer. They were then dehydratated in a graded ethanol series (30%, 50%, 70%, 90%, 95% and 100%) and embedded in Epon 812. The samples were sectioned on an ultra microtome, stained in 4% aqueous uranyl acetate, post-stained with lead citrate and examined with a Philips 201 electron microscope.

4. Conclusions

Cancer can be measured as a complex multistep pathology that requires the accumulation of several mutations giving to cells an aberrant proliferative advantage, improved resistance to pro-apoptotic stimuli and loss of differentiation markers. Increasing evidences underline the importance of the tumor microenvironment in the growth of cancer cells. Since it has been

shown to exert both pro- and anti-tumoral effects, the role of the immune system in fighting cancer progression has been contradictory. Due to the simplicity of genetic manipulations, *Drosophila* research could bring meaningful insights to our understanding of the mechanisms of communication between cancerous and normal cells, as well as between the tumor tissue and the immune system. Although it still remained unclear if the *Manf* gene from *Drosophila* is a true oncogene or a tumor-suppressor gene, we assumed that *DmManf* gene could play a particular function in the process and probably other additional mutations are simultaneously needed for the cancerous phenotype to become visible. Efforts to use *Drosophila* to explore issues specific to cancer will keep on growing. *Drosophila* is being used for what it does best: identifying novel oncogenes and tumor suppressors, and linking cancer-related genes together into complex signaling pathways [9]. The use of whole organisms *in vivo* is generally considered as being essential for understanding the tumorigenesis.

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